The Oxidation of *Octopus vulgaris* Hemocyanin by Nitrogen Oxides†

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ABSTRACT: The reaction of *Octopus vulgaris* hemocyanin with nitrite was studied under a variety of conditions in which the green half-met derivative is formed. Analytical evidence shows that the amount of chemically detectable nitrite in various samples of the derivative is not proportional to the cupric copper detected by EPR. The kinetics of oxidation of hemocyanin as a function of protein concentration and pH, in the presence of nitrite and ascorbate, is consistent with a scheme in which NO_2 is the reactive oxidant. We suggest that the green half-methemocyanin contains a metal center with one cuprous and one cupric copper without an exogenous nitrogen oxide ligand.

Hemocyanins are oligomeric O_2-transport proteins found in the hemolymph of several invertebrate species belonging to the phyla Mollusca and Arthropoda. Reversible O_2 binding occurs at a binuclear or type 3 copper site found in a hydrophobic pocket in the interior of the protein (Linzen et al., 1985). Spectroscopic studies suggest that the copper is cuprous in deoxy-Hc[1] and cupric in the oxy form (Brown et al., 1980). Resonance Raman spectroscopy shows that bound oxygen is in the form of peroxide, and therefore oxygen binding to deoxy-Hc is believed to involve a two-electron transfer from both Cu(I) ions to dioxygen. The oxygenated site is therefore depicted as a binuclear structure with a μ-dioxo bridge: [Cu(II)-O_2^{2-}-Cu(II)] (Freedman et al., 1976; Thamann et al., 1977). Deoxy-, oxy-, and the fully oxidized met-Hc are EPR silent. An endogenous ligand, thought to bridge the two metal ions in oxy- and met-Hc (Brown et al., 1980; Solomon, 1981), is believed to contribute to the large antiferromagnetic coupling of the cupric ions in both forms. X-ray diffraction studies at 3.2-Å resolution for an arthropod Hc (Gaykema et al., 1984) indicate that each metal ion is coordinated by three histidines without any bridging ligand.

Many attempts have been made to oxidize the copper in Hc. Oxidizing agents with high redox potentials such as ferricyanide and cyanomolybdate are ineffective, suggesting that the active site is strongly shielded from interactions with these relatively large anions. It is also thought that the hydrophobic character of the metal binding pocket may play a role in preventing or modulating the interaction with, or the release of, charged species (Klotz & Klotz, 1955).

Nitric oxide has been reported to react with Hc to produce a green derivative containing cupric copper (Schoot Uiterkamp, 1972). This same derivative, which has an EPR signal intensity accounting for 50% of total copper, can also be obtained by incubating deoxy-Hc with NO_2^-, in the presence or absence of ascorbate. It was suggested that NO is a copper ligand in this derivative (Schoot Uiterkamp, 1972). Van der Deen and Howing (1977), however, failed to observe any effect of isotopic substitution in the EPR spectrum of the derivative prepared with [^{14}N,^{15}N]nitrite. Also, infrared spectroscopy did not reveal an NO stretching frequency. Verplaetse et al. (1979) found 1 equiv of NO/equiv of EPR-detectable Cu(II) and suggested that NO was bound to a cuprous copper in this half-met derivative, with the adjacent cupric copper giving rise to the EPR signal.

A different description of the green product was given by Himmelwright et al. (1978) and Solomon (1981), who suggested that the half-met derivative binds an NO_2^- group, produced through reaction of NO with an intermediate, EPR-undetectable met-Hc. Here, nitrite was thought to be a tightly bound bridging ligand between Cu(II) and Cu(I), bound to the coppers through oxygen rather than nitrogen atoms. However, in a recent study, the binding constant for nitrite with methemocyanin (Tahon et al., 1988) was shown to be so small making it unlikely that nitrite is tightly bound to copper in the semimet derivative.

In each of the cases described above, the green half-met product is suggested to contain an exogenous nitrogen oxide ligand, either NO or NO_2^-, bound to a copper ion. The lack of agreement upon the structure, however, called for a reexamination of the product and its formation. We have therefore undertaken a reinvestigation of the mechanism of formation of the green half-met Hc and the stoichiometry of exogenous nitrogen oxide ligands. We show that the amount of nitrogen oxide detected by chemical analysis is much less than stoichiometric with EPR-detectable cupric copper and suggest that structures previously proposed for the oxidized copper site are untenable.

Materials and Methods

Hemocyanin was purified from the hemolymph of live *Octopus vulgaris* collected at the Stazione Zoologica di Napoli (Naples, Italy) (Salvato et al., 1979). Reagents were of the best grade commercially available and were used without further purification. The concentration of purified Hc was determined spectrophotometrically (E_{278} = 1.41 (mL mg^{-1} cm^{-1} in 0.1 M Tris-HCl, pH 8.0) (Salvato et al., 1979) and may be converted to molarity units by using a molecular weight of 50 000 for the subunit containing a single binuclear copper site. The fraction of protein in the oxygenated form was

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[1] Abbrevations: Hc, hemocyanin; EPR, electron paramagnetic resonance.

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calculated from the absorbance ratio A_{348}/A_{278}, which equals 0.25 for the fully oxygenated protein (Salvato et al., 1974).

Optical absorption and circular dichroism spectra were recorded on a Perkin-Elmer Lambda 5 spectrophotometer and a Cary 61 spectropolarimeter, respectively. Copper concentration was measured by atomic absorption using a Perkin-Elmer 4000 flame spectrometer. Fluorescence spectra were recorded at 21 °C by using a Perkin-Elmer Model 44 instrument operating in the "ratio" mode in order to correct for lamp fluctuations. Solutions had an absorbance at the excitation wavelength (294 nm) less than 0.06 to minimize errors due to inner filter effects. Emission quantum yield was calculated as previously reported (Ricchelli et al., 1984).

EPR spectra were recorded at 140 K on a Bruker ER200 spectrometer. Spin quantitation was carried out by double integration of EPR spectra using Cu(II)-EDTA and purified Cu_2Zn_2 superoxide dismutase as spin standards.

Preparation of Green Half-Met-Hc. A concentrated solution of Hc (50–75 mg/mL; 2–3 mM Cu) was dialyzed at 6 °C for 20 h against 50 mM phosphate buffer, pH 5.5, containing 5 mM $NaNO_2$ and 5 mM ascorbate. Excess reactants were then removed by dialysis of the protein sample against 20 mM phosphate buffer, pH 7.0. The derivative could also be prepared under anaerobic conditions with or without ascorbate (i.e., with continuous bubbling of N_2 through the dialysis buffer).

Assay of Nitrite. The green half-met derivative was analyzed for its content of nitrogen oxide ligand (determined as NO_2^-) by using the sulfanilic acid method (Vogel, 1961) as follows: Aliquots of Hc solution (20–100 μL) were added to 100 μL of sulfanilic acid (0.035 M in 2.4 M hydrochloric acid), and after 6 min, 200 μL of α-naphthylamine (0.034 M in 0.15 M hydrochloric acid) and 200 μL of sodium acetate (1.2 M) were added. The solution was then brought to 6 mL with water and was incubated for 30 min at room temperature. The protein precipitate that forms during the procedure was removed by centrifugation at 3000 rpm for 15 min. The concentration of nitrite was obtained from the absorbance at 520 nm on the basis of a comparison with a calibration curve prepared from replicate analyses using sodium nitrite (Merck Darmstadt) as a standard. The solutions used for the standard curve contained native Hc, included to remove possible interferences. No interference was found in separate determinations of nitrite when aliquots of NO_2^- standard solution were added to half-met-Hc.

The sulfanilic acid method for the assay of nitrite was also shown to be useful for other oxides of nitrogen. For instance, NOFe(II)-tetraphenylporphyrin prepared according to Scheidt and Frisse (1975) when dissolved in dimethyl sulfoxide gave the expected yield of chromophore in the assay described above based on the reaction:

$$2NO + O_2 + H_2O \rightarrow HNO_3 + HNO_2$$

Kinetic Measurements. The kinetics of oxidation of oxy-Hc to the green half-met form was followed optically and by EPR spectroscopy. A concentrated solution of protein (approximately 1 mM in copper) was divided into several aliquots which were dialyzed at 20 °C against 50 mM phosphate buffer, pH 5.5, containing 5 mM $NaNO_2$ and 5 mM sodium ascorbate. Dialyses were performed by using 2-mm diameter Servapore (Serva) dialysis tubes in order to minimize the diffusion time of solutes. The Hc samples were analyzed for total copper, EPR-detectable copper, and residual absorbance at 348 nm as a function of time. The total copper measured by atomic absorption was accounted for by a material balance of the copper in half-oxidized sites plus the copper remaining in native (oxy-) Hc detected optically.

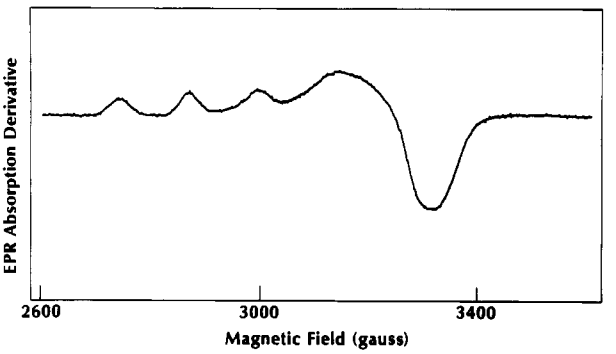

FIGURE 1: EPR spectrum of *O. vulgaris* half-met-Hc. Microwave frequency, 9.426 GHz; modulation amplitude, 5 G; T = 140 K.

Table I: Quantitation of Nitrogen Oxide Species and EPR-Detectable Copper in Samples of Half-Met-Hc

	EPR-detectable Cu(II) (mM)	nitrite (mM)
half-met-Hc[a]	0.394	0.011
anaerobic preparations	0.597	0.039
	0.626	0.059
	0.700	0.063
aerobic preparations	0.478	0.133
	0.910	0.227

[a] Hc was prepared as described under Materials and Methods.

RESULTS

Chemical and Spectroscopic Properties of Half-Met-Hc. O. vulgaris oxy-Hc is converted from the native blue protein to the green half-met derivative upon dialysis against an aerobic solution containing 5 mM $NaNO_2$ and 5 mM ascorbate in 50 mM phosphate buffer, pH 5.5. The product exhibits an EPR signal that arises from a single paramagnetic species whose spectrum, shown in Figure 1, is indistinguishable from that previously published (Shoot Uiterkamp, 1972). The EPR signal intensity measured upon completion of the oxidation reaction corresponds to 50% of the total copper in the sample. Neither exhaustive dialysis of the derivative against phosphate buffer nor the addition of a 10-fold molar excess of $NaNO_2$ changes the intensity or the line shape of the spectrum. Regeneration of the native oxy form can be readily accomplished by treating the green derivative with hydroxylamine (100 mM). Sodium dithionite (100 mM) is also effective, though at a much slower rate.

Analytical determination of nitrogen oxide in anaerobic preparations of half-met-Hc, which were exhaustively dialyzed against phosphate buffer, always gives values less than 0.1 mol of nitrite/mol of oxidized copper (Table I). The nitrite content is higher in the derivative prepared aerobically yet is far from stoichiometric with EPR-detectable cupric copper. The variable amount of nitrite is not related to the line shape of the EPR spectrum, which is the same in samples from different preparations.

The absorption and circular dichroism spectra of the green derivative are shown in Figure 2. The absorption spectrum is characterized by a broad asymmetric band with a maximum near 670 nm. The extinction coefficient is relatively high (E_{670} = 500 M^{-1} cm^{-1}), indicating a nontetragonal geometry, which is in accord with the EPR line shape. The broad optical absorption is actually the envelope of several bands, probably attributable to d–d transitions of the oxidized metal (Solomon, 1981). Several Cotton maxima are evident in the CD spectrum, with positive and negative ellipticities extending to the near-infrared region. A strong negative band around 330 nm

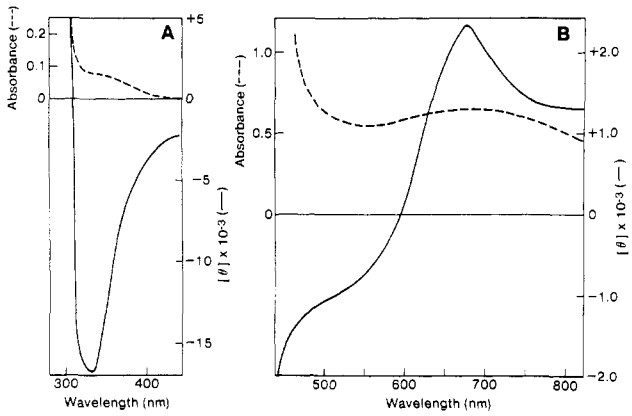

FIGURE 2: Optical absorption (dashed line) and circular dichroism (solid line) spectra of *O. vulgaris* green half-met-Hc in the near-UV-visible (A) and visible (B) region.

is observed, which corresponds to a small shoulder in the absorption spectrum. These features are characteristic of the green half-met-Hc derivative and are not due to unreacted starting material since the Cotton maximum is approximately 20 nm blue shifted compared to that for oxy-Hc (Tamburro et al., 1977). The fluorescence quantum yield of the derivative was measured since this parameter is known to be dependent on the oxidation state of the metal center (Ricchelli et al., 1987). Green half-met-Hc has a quantum yield of 1.8%, which is intermediate between that of the oxy form (1.1%) and that of the deoxy form (3.3%) (Ricchelli et al., 1984). The fluorescence emission (excitation wavelength 294 nm) has a maximum at 332 nm, coincident with that of the other forms, which indicates preservation of the structural organization of the protein, despite the oxidation process.

Kinetic Measurements. Figure 3 shows the rate of oxidation of Hc (70 mg/mL) monitored by the change in optical absorbance at 348 nm and by the appearance of the EPR signal attributable to cupric copper. The absorbance data are referred to the initial value for native Hc at 348 nm and are reported as a percentage of this absorbance. The signal amplitude in the *g*-perpendicular region for a sample of half-met-Hc prepared as described under Materials and Methods, which contained a known amount of copper, was used as "100%". In the presence of nitrite and ascorbate in air (Figure 3A), conversion to the half-met derivative is complete in 6–7 h; the reaction proceeds more slowly under anaerobic conditions (Figure 3B). The process can be described by a (pseudo-)first-order rate constant k_{app}, which decreases from 1.3 $\times 10^{-4}$ to 6.5 $\times 10^{-5}$ s^{-1} when O_2 is absent (Figure 3A′,B′). In the absence of both O_2 and ascorbate (Figure 3C), the reaction proceeds to 50% conversion to the half-met form. In this case also, the semilogarithmic plot yields a straight line (if one assumes that an equilibrium between Hc and half-met Hc is reached), yielding a k_{app} value of 2 $\times 10^{-4}$ s^{-1} for the initial rate (Figure 3C′).

As shown in Figure 3A (and B), the loss of oxy-Hc corresponds to the increased intensity of the EPR signal. A plot of optical change versus EPR signal intensity, based on the data of Figure 3, is shown in Figure 4. The plot is linear, with a slope of unity and an intercept at the origin. The formation of EPR active species occurs at the expense of the 348 nm absorbing material in each of the experiments described above (Figure 3A,B,C).

The disappearance of 348-nm absorbance due to native Hc (Hc = 3 mg/mL) was also followed as a function of ascorbate concentration with 5 mM NO_2^-, at pH 5.5 and 6.1. In each case (panels A and B of Figure 5), the rate increases nonlin-

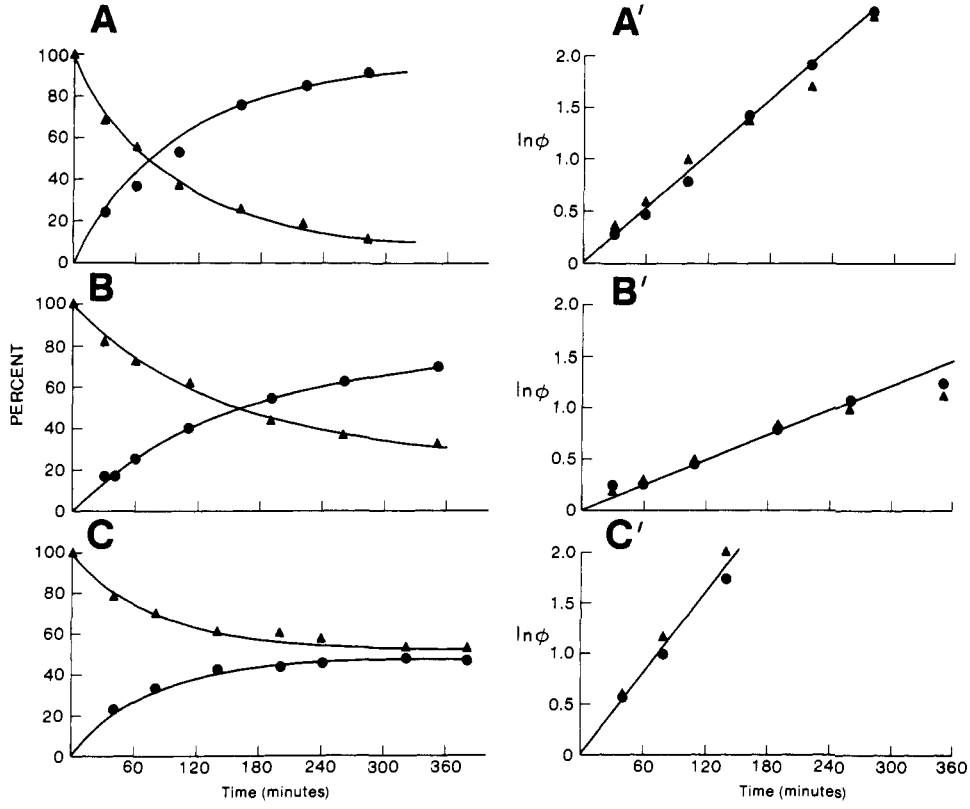

FIGURE 3: Conversion of oxy-Hc to green half-met-Hc. The fractional appearance of EPR-detectable half-met-Hc (●) and disappearance of 348-nm absorption (▲) are plotted as a function of time. The reaction conditions are as follows: (A) 5 mM nitrite, 5 mM ascorbate, aerobic; (B) 5 mM nitrite, 5 mM ascorbate, anaerobic; (C) 5 mM nitrite, anaerobic. In A′, B′, and C′ the results from A, B, and C are presented in semilogarithmic plots. In C′, the data are reported taking into account a limiting value of EPR-detectable half-met-Hc and residual absorbance at 348 nm of 50%. For details, see Materials and Methods.

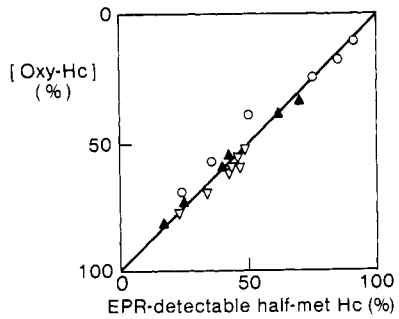

FIGURE 4: Correlation between the percent of EPR-detectable half-met-Hc and percent of remaining oxy-Hc. The symbols (○), (▲), and (▽) refer to data obtained respectively under conditions A, B, and C of Figure 3.

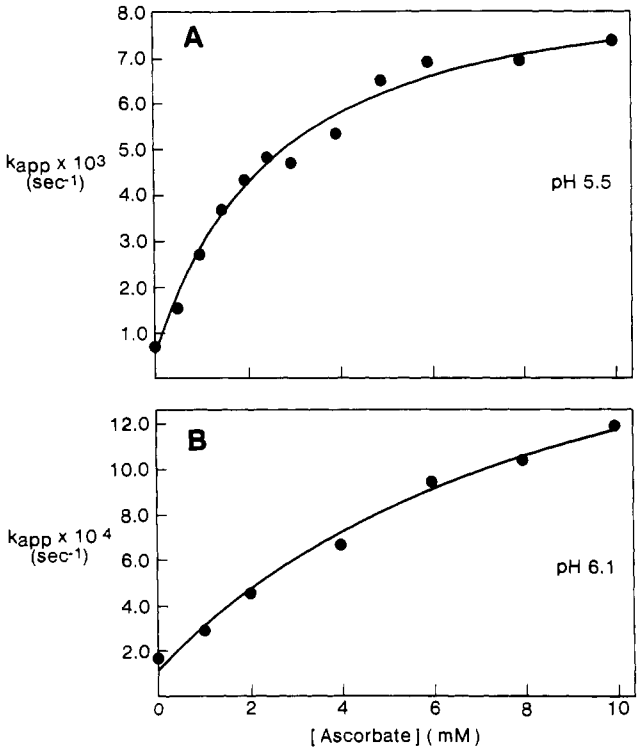

FIGURE 5: Pseudo-first-order rate constant of Hc oxidation versus ascorbate concentration. Experiments were carried out in 20 mM phosphate buffer containing 5 mM nitrite throughout, at pH 5.5 and 6.1 as labeled. For details, see Materials and Methods.

early with increasing ascorbate concentration, the curvature being more pronounced for the experiment at pH 5.5. The dependence of k_{app} on nitrite concentration is linear at pH 5.5 (Figure 6).

The value of k_{app}, determined in the presence of 5 mM NO_2^- and 5 mM ascorbate, shows a marked decrease as pH increases (Figure 7A). This behavior suggested that the oxidation rate was dependent on the concentration of undissociated HNO_2. In fact, a linear relationship was found between k_{app} and the fraction of undissociated HNO_2, using $pK_a = 3.37$ (Figure 7B).

The rate of oxidation of hemocyanin in experiments carried out at low protein concentration was higher than at high protein concentration in 5 mM nitrite and 5 mM ascorbate, at pH 5.5: an increase from 1 to 6.0 mg of Hc/mL causes a 5-fold decrease in k_{app}.

The oxidation of Hc was also attempted by using NO. The gas was generated in a Thunberg tube by the addition of ascorbic acid (0.5 M in 0.1 M HCl) to a 0.5 M solution of $NaNO_2$. NO was withdrawn from the gas phase with a gas-tight syringe after evolution had proceeded long enough to eliminate O_2 from the system. No reaction of Hc with NO

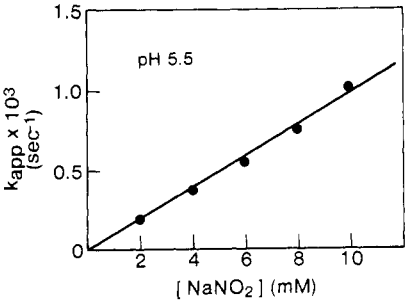

FIGURE 6: Pseudo-first-order rate constant of Hc oxidation versus nitrite concentration. Experiments were carried out in 20 mM phosphate buffer, pH 5.5. For details, see Materials and Methods.

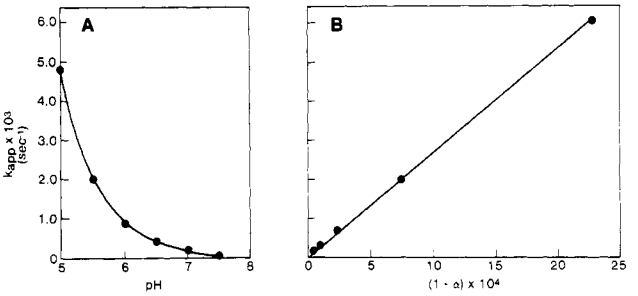

FIGURE 7: Oxidation of Hc in 5 mM nitrite and 5 mM ascorbate. (A) pH dependence of pseudo-first-order rate constant. Buffer containing 5 mM nitrite and 5 mM ascorbate was used throughout. (B) Pseudo-first-order rate constant versus concentration of undissociated HNO_2. This is calculated from the data in (A) by using $pK_a = 3.37$ for the HNO_2/NO_2^- pair.

occurred when 50 μL of NO was injected into a tonometer containing 4 mL of Hc solution (2 or 20 mg/mL) under an N_2 atmosphere. Upon introduction of air, however, there was a rapid conversion of Hc to the half-met form, either at pH 5.5 or at pH 7.0.

Discussion

The reaction of *O. vulgaris* Hc with nitrite and ascorbate according to a new method described here yields a derivative that exhibits the same optical and EPR properties as green half-met hemocyanin preparations obtained in other laboratories using different procedures. The product is proposed to be a half-met form of the protein containing Cu(II) and Cu(I) in an active site that is free of exogenous nitrogen oxide ligand. Convincing support for this conclusion is the analytical data which show that the amount of bound oxide of nitrogen released upon acid treatment (measured as nitrite) represents only a fraction of the oxidized copper detected by EPR.

It is surprising that the same green oxidation product is formed under a large variety of experimental conditions. The same species is produced by using nitrite (Schoot Uiterkamp, 1972; Verplaetse et al., 1979), nitrite plus ascorbate (Schoot Uiterkamp, 1972; Van Der Deen & Howing, 1977; Himmelwright et al., 1979), nitric oxide, or nitrogen dioxide (Schoot Uiterkamp, 1972; Van Der Deen & Howing, 1977; Verplaetse et al., 1979). One can interpret this phenomenon either by assuming that the various nitrogen oxide compounds interact at the Hc active site with different mechanisms or by suggesting that the same oxidant is formed from different precursors.

Early studies addressing the reaction of NO with Hc were carried out in mixtures of nitrite and ascorbate with the assumption that NO produced in situ was responsible for the oxidation of Hc to form the green, half-met product; later, a direct addition of NO to the protein was found to be effective (Schoot Uiterkamp, 1972). We have shown, however, that,

under strictly anaerobic conditions and at low NO concentration, no reaction occurs. It is unlikely, then, that NO is the immediate oxidant given the additional fact that the aerobic reaction of Hc with nitrite and ascorbate is much faster than the anaerobic reaction. (Aerobically, NO levels are expected to be vanishingly low.)

Nitrite anion may be ruled out as the oxidant since we observe a strong decrease in the rate of oxidation with increasing pH. The pH dependence, therefore, suggests that HNO_2 might be the active oxidant. However, when Hc is exposed to NO_2 generated in situ via reaction of NO with O_2, oxidation occurs much faster than in the dialyses against excesses of NO_2^- and ascorbate, either at pH 5.5 or at pH 7.0.

We suggest that NO_2 is the active oxidant in the reactions of Hc with various nitrogen oxide reagents. NO_2 is a species that is always present in solutions containing NO and is formed either by oxidation of NO by O_2 or by dismutation of NO in the absence of O_2. As NO_2 would arise from nitrite, one expects the overall reaction rate to be dependent on the concentration of nitrite, as is found. The inverse dependence of the rate of oxidation with nitrite and ascorbate on protein concentration suggests that the steady-state level of NO_2 in dialysis mixtures is low. At high protein concentration, the reaction apparently follows first-order kinetics, the rate constant representing the production rate of NO_2. At low protein concentration, the reaction displays second-order kinetics.

The kinetics of oxidation shown in Figure 3 indicate essential differences between the anaerobic reaction with nitrite and the reaction when both oxygen and ascorbate are present. Aerobically, NO_2 is formed by direct oxidation of NO, the latter arising from the reaction of NO_2^- and ascorbate. Anaerobically, NO_2 originates solely from dismutation of NO. The leveling off of the rate of reaction (Figure 3C) in the presence of NO_2^- but in the absence of both ascorbate and O_2 suggests an inhibitory effect mediated by dismutation products or an inhibition by one of the intermediates in their formation. (The role of ascorbate might also be related to the elimination of inhibiting species formed in the anaerobic reactions.) The high initial reaction rate observed in the absence of O_2 and ascorbate could involve an oxidation of deoxy-Hc by HNO_2, and the mechanism could follow a Haber–Weiss scheme:

$$[\text{Cu(I) Cu(I)}] + HNO_2 \rightarrow [\text{Cu(I) Cu(I)–ONOH}] \rightarrow [\text{Cu(I) Cu(II)}] + NO + OH^-$$

Finally, we find that, in aerobic reactions, some nitrite is found in the half-met derivative though its molar ratio is far less than stoichiometric with oxidized copper. The nitrite detected is suggested to reside in nitrous esters formed in reactions on protein side chains:

$$ROH + HONO \rightarrow R\text{–}ONO \text{ (or } R\text{–}NO_2)$$

The derivatization of the polypeptide was not specifically addressed in previously published work and, though apparently unavoidable, is probably minimized by the dialysis procedures we have employed in which the concentration of reagents is kept low. At neutral pH the side products are rather stable but hydrolyze rapidly at the acidic pH required for nitrite determination by the sulfanilic acid method.

As the stoichiometry of bound nitrogen oxide species is far less than stoichiometric with oxidized copper, previously proposed structures involving metal ligation to these species are not well founded.

Acknowledgments

This work is dedicated to the memory of our deceased colleague Marcello Alviggi, who participated in the early stages of this investigation. We acknowledge Dr. Agostini for his expertise in EPR spectroscopy and Dr. William Blumberg for helpful discussion and criticism. We also acknowledge the technical assistance of A. Cervellin in the Hc preparation.

Registry No. NO_2^-, 14797-65-0; Cu, 7440-50-8; NO_2, 10102-44-0.

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